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13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)
This proposal examines the novel concept that H_2O_2 generating oxidase-mediated reactive oxygen species in breast epithelium contribute to the development of breast cancer. Constructs to express xanthine oxidase under the direction of mouse mammary tumor virus (MMTV) promoter were generated. Transfections performed to generate cell lines stable expressing the enzyme were unsuccessful hence urate oxidase was used as an alternative . The choice for urate oxidase is based on the fact that we have used this enzyme in previous studies and showed that cells expressing this enzyme reveal the characteristic crystals of this enzyme in peroxisomes. Transgenic mice expressing UOX under the transcriptional control of MMTV promoter were generated. We have injected the construct in fertilized ova and identified five founder mice initially which failed to transmit the transgene. Additional microinjections generated 9 founders. Five of these were found to express the transgene. Southern, Northern and Western analyses showed the presence of the transgene in the mammary, and testicular tissues. We will now initiate studies to examine the role of reactive oxygen species in causing cell death, cell proliferation and neoplastic transformation. In parallel, we are also developing a stable cell line in MCF10A (a non tumorogenic cell breast epithelial cells) expressing UOX under the control of MMTV promoter. UOX expressing non tumorogenic cells will be injected into nude mice to develop into carcinomas.

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Anjana Yeldandi 7-1-02

PI - Signature Date

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INTRODUCTION

Carcinoma of the breast is overwhelmingly a disease of females. In the United States, the age-standardized incidence of breast cancer has doubled during the past four decades, and it is estimated that there will be over 200,000 new cases of breast cancer by year 2000. The established risk factors are both non-hormone mediated and hormone-mediated. Oxygen free radicals are a well-established risk factor for cancer and aging (1-4). Evidence for the role of free radical induced DNA damage in aging, and cancer comes from the correlations between high consumption of fruit and vegetables, or of specific dietary antioxidants and a relatively low incidence of several types of cancers (5-8). Recent literature suggests a role for free radical induced injury in the development of breast cancer (9-16). The idea of free radical induced injury having a role in breast cancer development is intriguing since it opens up the possibility of antioxidants being able to prevent its development.

Our novel idea or hypothesis is that the increased incidence of breast cancer in the United States is due to increased generation of reactive oxygen species (ROS) in the breast epithelium during the reproductive period and antioxidant activity will be beneficial in preventing breast cancer.

Specifically, our provocative idea, which is based on sound biological basis, is that xanthine oxidase, an enzyme which converts xanthine to uric acid and in the process utilizes molecular oxygen as the electron acceptor and releases substantial amounts of ROS, is responsible for neoplastic transformation in the breast. Xanthine oxidase/xanthine dehydrogenase is a multifunctional protein, which catalyzes the last two steps in purine metabolism in man, forming the end product uric acid (hypoxanthine to xanthine; and xanthine to uric acid). This enzyme is a homodimer with a subunit molecular mass of ~ 150 kDa; the dehydrogenase form utilizes NAD+ as the electron acceptor, and is converted into oxidase both *in vivo* and *in vitro*, initially through thiol group oxidation, and subsequently irreversible conversion with proteolytic cleavage of an approximately 20 kDa fragment from each subunit. This proteolytic cleavage is mediated presumably by a calcium-dependent protease. Xanthine oxidase reacts with molecular oxygen to produce superoxide radicals and H_2O_2. Historically, xanthine oxidase was first isolated from the cow's milk in 1975 and the cDNA encoding the human xanthine

oxidase/dehydrogenase has been cloned recently. We have obtained a full-length human xanthine oxidase cDNA from Professor Kari Raivio (Helsinki, Finland), and have expressed recombinant human xanthine oxidase in insect cells using the baculovirus expression system We also obtained antibodies generated against human xanthine oxidase from Prof. Raivio and localized this enzyme in the lactating epithelium of human breast and in the lactating breast epithelium of mouse (17).Thus, our idea is based on the notion that xanthine oxidase, which is present in milk for possible antimicrobial activity, to keep the milk sterile, plays havoc with the breast epithelium of women at risk. We will test the idea that xanthine oxidase over-expression in breast epithelium leads to neoplastic transformation using in vitro and *in vivo* transgenic approaches. For this purpose stably transfected mammary epithelial cells will be generated and exposed to xanthine to produce H_2O_2/ ROS and transformation potential will be assessed. Likewise, we will generate transgenic mouse lineages over-expressing xanthine oxidase under the control of mouse mammary tumor virus (MMTV) promoter. We have considerable past experience *in vitro* transformation work using peroxisomal fatty acyl-CoA oxidase and peroxisomal urate oxidase in African green monkey kidney cells and we also have the expertise in our laboratories to generate transgenic mice (18-21). Tamoxifen, a well-established chemotherapeutic agent in breast cancer has antioxidant properties and its role in preventing lipid peroxidation has been suggested to be beneficial in chemoprevention (22,23). Tamoxifen has also been reported to suppress formation of ROS by human neutrophils (24,25). This property could contribute to its anticarcinogenic action by preventing hydroxyl radical-mediated DNA damage.

BODY

Funding period: 6-1-99 to 6-30-02.

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Cao, Wendy	1-24-00	to	2-04-01.

Specific Aim 1: *Overexpress xanthine oxidase in non-tumorigenic human mammary epithelial cells (MCF-10A) under the control of MMTV-LTR promoter and ascertain the development of neoplastic transformation when exposed to the substrate xanthine.*

To overexpress, xanthine oxidase (XOX) in MCF-10A, we have cloned the entire coding sequence of XOX in pMSG vector, which expresses the inserted cDNA under the control of MMTV promoter. Plasmid DNA was transiently transfected into MCF-10A cells and stable transformants were selected with media containing GPT (xanthine , mycophenolic acid, aminopterin, thymidine and hypoxanthine). Due to expression problems. We had to switch over from XOX expression to UOX expression. UOX was also expressed under the control of MMTV promoter and stable cells were generated with the help of GPT selection. Characterization of the protein expression is under progress along with the generation of stable cell lines. MCF-10A cells expressing UOX will be selected and ascertained for the neoplastic transformations upon injection into nude mice.

Specific Aim 2: *Generate transgenic mouse lineages that overexpress xanthine oxidase under the control of MMTV-LTR promoter and utilize this in vivo model to explore the role of xanthine oxidase-generated ROS in the development of breast cancer*

We are testing the idea that XOX or UOX overexpression in breast epithelium leads to neoplastic transformation using *in vitro* and *in vivo* transgenic approaches. Our proposal will test the idea that XOX mediated generation of ROS in breast epithelium contributes to the development of breast cancer and that XOX levels are hormonally regulated, with highest enzymatic activity in breast epithelium during the reproductive phase of female biology. The hypothesis that breast cancer is due to ROS generated by XOX, or other H_2O_2-generating oxidases such as UOX, will be tested using molecular genetic approaches. The specific aims of the proposed study will address the fundamental issues related to our idea/hypothesis regarding the role of ROS in breast cancer pathogenesis.

Construction of Urate oxidase (UOX) expression plasmid under the control of MMTV promoter:

Rat UOX cDNA, previously cloned in our laboratory was used as template to PCR amplify the coding region and cloned into MMTV promoter plasmid at the EcoR I sites of exon 3 region as shown in the Fig. 1. The plasmid contains exon 2 and exon 3 of rabbit beta globin genomic DNA, under the control of MMTV promoter. The plasmid was sequenced and verified for mutational errors and the entire cassette consisting of MMTV promoter, Exon 2 and Exon 3 of rabbit beta globin, rUOX along with Poly A tail was released with double digestion of Hind III and XhoI restriction enzymes. This cassette was used for the generation of chimera mice.

Generation and Characterization of transgenic mice expressing urate oxidase:

We have successfully microinjected the cassette containing MMTV-rUOX into the fertilized ova and generated transgenic mice by implanting these ova in pseudo-pregnant mice. Analyses of 2-week old mouse-tail DNA was performed by Southern blotting and by polymerase chain reaction of partial cDNA of 300 bps. We have

previously reported the identification of 5 founders (3 males and 2 females), but these mice were found to be either unproductive or failed to transmit the rUOX transgene to their siblings (Fig. 2). Further injection of ova to generate more founders were taken up and subsequent analyses of the pups generated showed 9 more pups carrying the transgene (Fig 3). These founders (males designated as □ and females designated as O) were bred with wild type male or female mice .

Transgenic analyses of the offspring were carried out by tail genomic DNA analyses which was digested with EcoRI restriction enzyme and Southern blot analyses (Tail genomic DNA was isolated and after restriction digestion was blotted onto a nylon membrane and probed with linearized and labeled UOX cDNA probe). Out of the 9 founders, two founders were found to be non-reproductive (Nos. 189 and 197, designated with X) and also 2 founders did not transmit the transgene to their offspring (Nos. 189 and 201, designated with XX). 5 founders successfully transmitted the gene to their offspring (Fig. 3). These heterozygous offspring (either from the same parent/founder, or from different founders, are shown in the fig. 3) were bred to generate homozygous offspring, in order to boost transgenic expression. A representative Southern analyses is shown in Fig. 4.

Heterozygous were analyzed for UOX expression. Northern analyses showed UOX mRNA expression in testis (Fig.5b) and in Breast (Fig.5d). UOX expression in liver is shown as control (Fig 5a and c). Western analyses confirmed the presence of UOX protein (Fig. 6).

rUOX exists as as typical rod like cylindrical crystalloid structures. In order to distinguish rUOX from mUOX, we fixed mammary and testicular tissues in paraformaldehye and processed them for electron-microscopy fixation. Analysis of both testicular and mammary tissues showed the presence of typical rUOX structures though many more in testis then in mammary tissues. Electron microscopic sections of different magnifications are presented in Fig.7a, b and c for testis and Fig. 8a, b and c for mammary tissues, confirming the rod like crystalloid structures in both the tissues indicated by the arrows.

Expression of rUOX was localized in testis and mammary tissue using immuno histochemistry and insitu hybridization. Antibodies developed against the UOX cross reacted with the expressed protein in the cytoplasm of the epithelial cells of mammary tissue and in the testicular tissue. (Fig.9a and 9b). RNA expression of rUOX was also localized in the epithelial cells and in the testicular tissue (Fig. 10a and 10b).

We have also transfected rUOX under the control of MMTV promoter and CMV promoter individually into MCF10A, a non tumorogenic breast epithelial cells. Clones expressing UOX were selected with mycophenolic acid (gpt selection media) for pMSG vector) and G418 (neomycin resitance) for pcDNA 3.1 vector. Individual clones were tested for RNA expression by northern blot analysis and protein expression by western blot analysis.. Clones are being amplified and readied to be injected into nude mice for development for carcinomas.

Specific Aim 3: *Manipulate the level of oxidative stress in in vitro and in vivo systems using tamoxifen. Since MCF-10A cell lines are estrogen receptor negative, any chemotherapeutic/protective effect seen will be attributable to the antioxidant property of tamoxifen.*

This aim could not be initiated due to the fact, there was delay in generating stable cell lines expressing, first with xanthine oxidase and later with urate oxidase.

In vivo models, expressing UOX in the breast, failed to show up any tumor formation or neoplastic transformation in breast or testicular tissue even under the dexamethasone influence.

Specific Aim 4: *Determine the expression of xanthine oxidase in human breast carcinomas and proliferative epithelial breast lesions using immunoperoxidase, northern analysis and in situ hybridization.*

Specific Aim 4 could not be initiated, since xanthine oxidase failed to express as mentioned in specific aim 1. Efforts are under way to screen the human samples with XOX once the data from in vivo and in vitro models of UOX becomes clear.

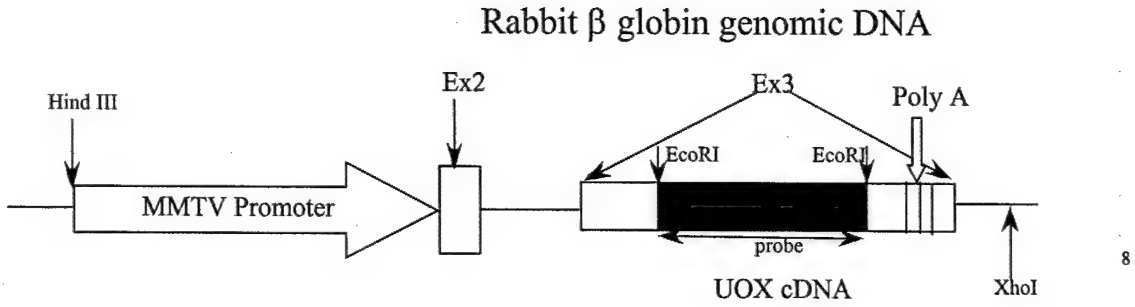

Fig.1 Transgene construct of MMTV-UOX

Fig.2: **MMTV-UOX**

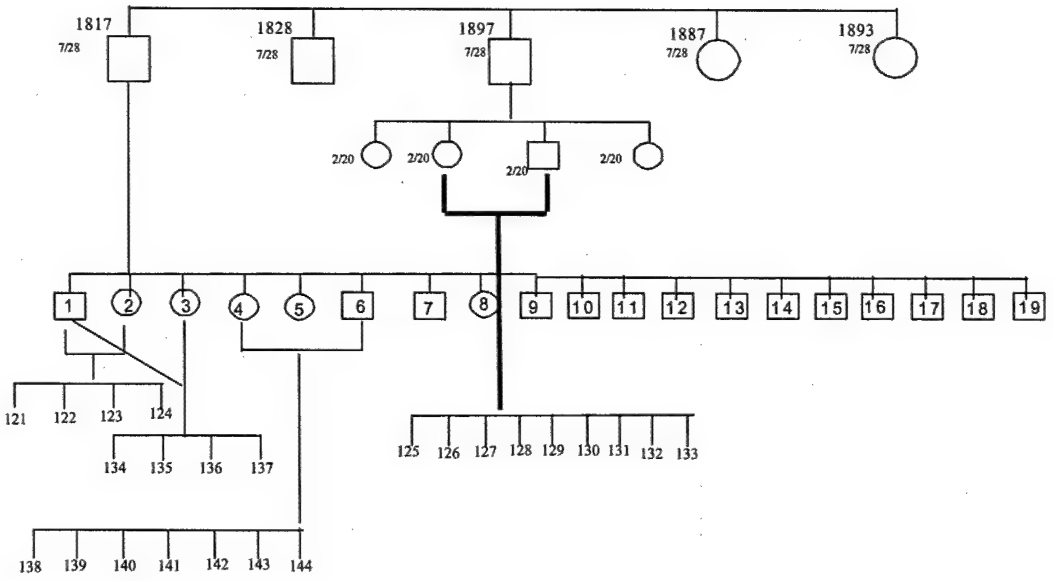

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Fig.3:

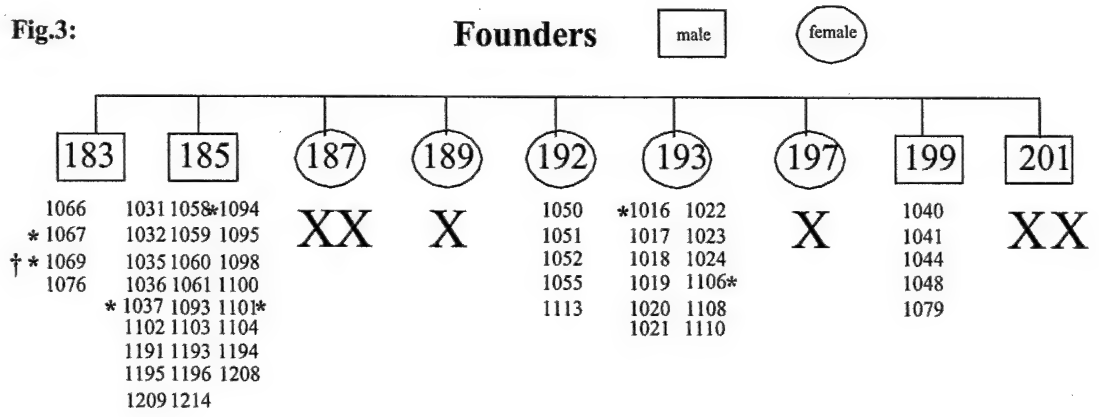

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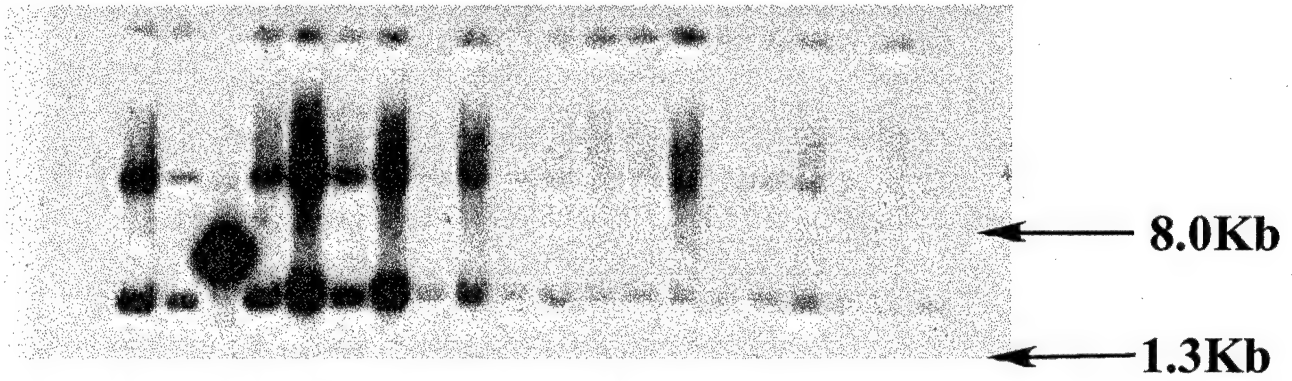
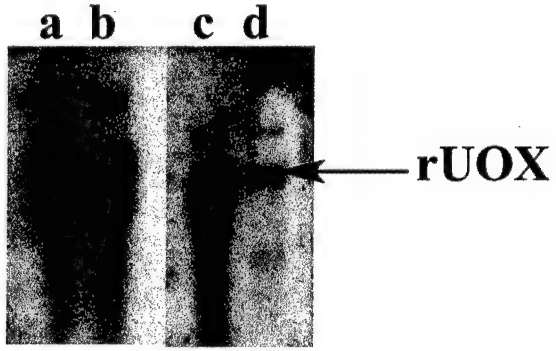
Fig.4



Southern Blot analysis of transgenic mice: mouse tail genomic DNA was isolated and restriction digested with EcoR I. The digested DNA was electrophoressed on 1% agarose gel and transferred to nylon membrane. The membrane after blocking was probed with labeled R UOX probe.

Fig.5

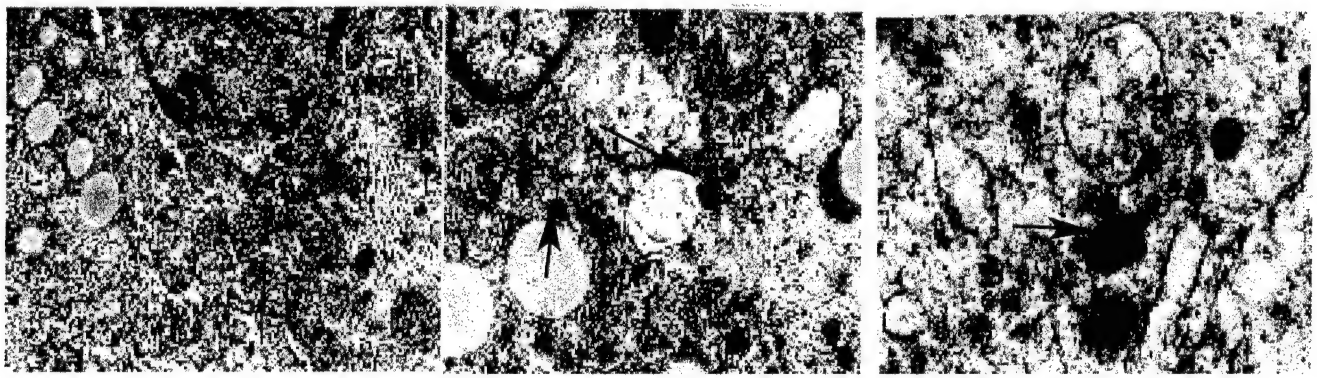


Northern blot analysis: Total RNA was isolated from testis, liver and mammary tissue and electrophoressed on a 1% agarose gel. Thegel was transferred to nitrocellulose membrane and probed with rUOX cDNA labelled probe and exposed to x-ray film. Liver:a &c, testis:b, mammary tissue:d.

Fig.6

Western blot analysis: UOX expressing tissue of mammary and testis, were homogenized in PBS and separated on a 10% SDS-PAGE. Proteins transferred on to nitrocellulose membrane was probed with anti uox antibody and developed with NBT/BCIP reaction.

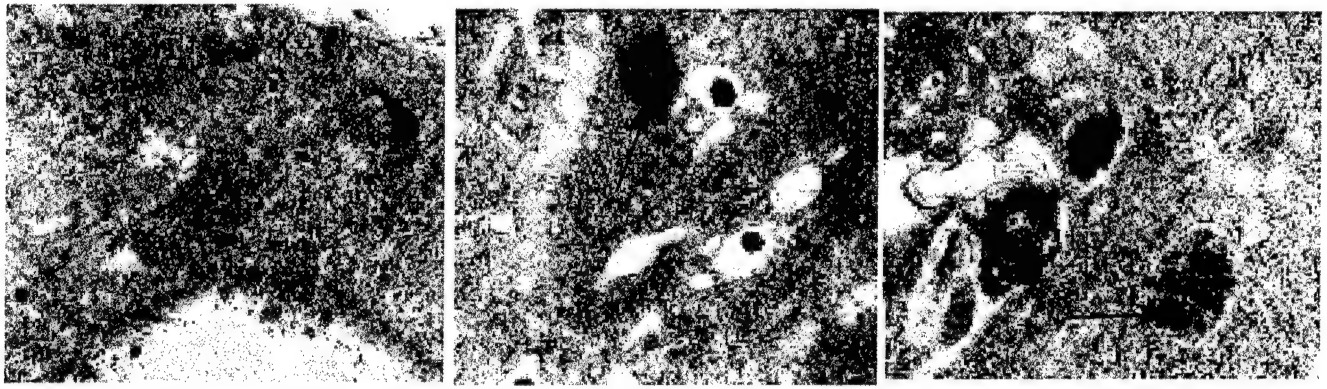
Fig.7



a b c

Electron micrographs for testicular tissue from transgenic mice expressing rUOX under the control of MMTV promoter, showing rod like UOX crystal structures, confirming the presence of UOX

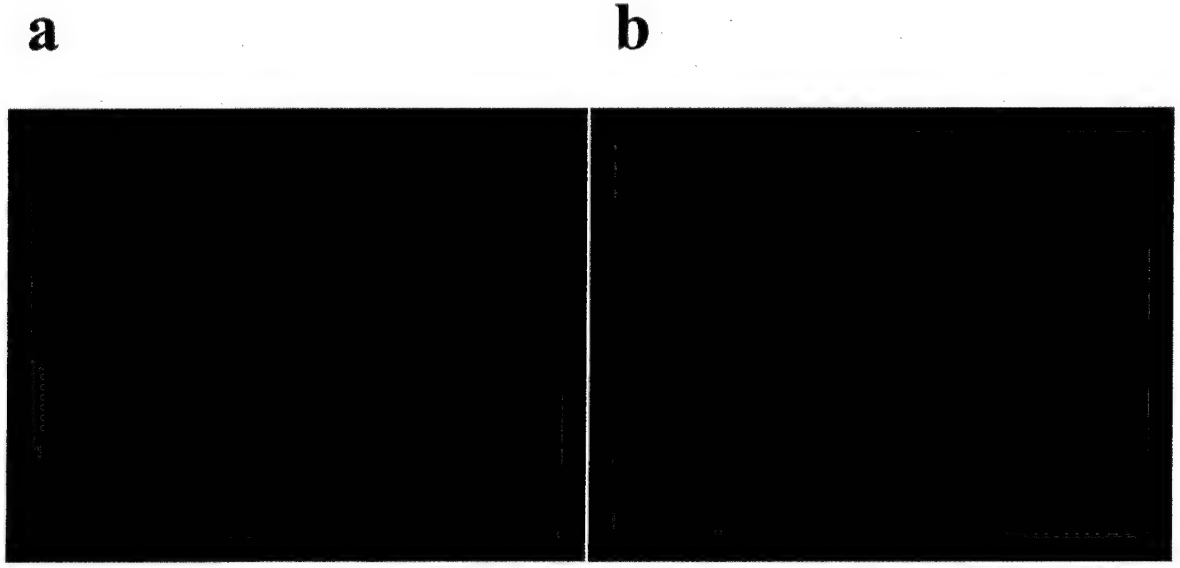
Fig.8



a b c

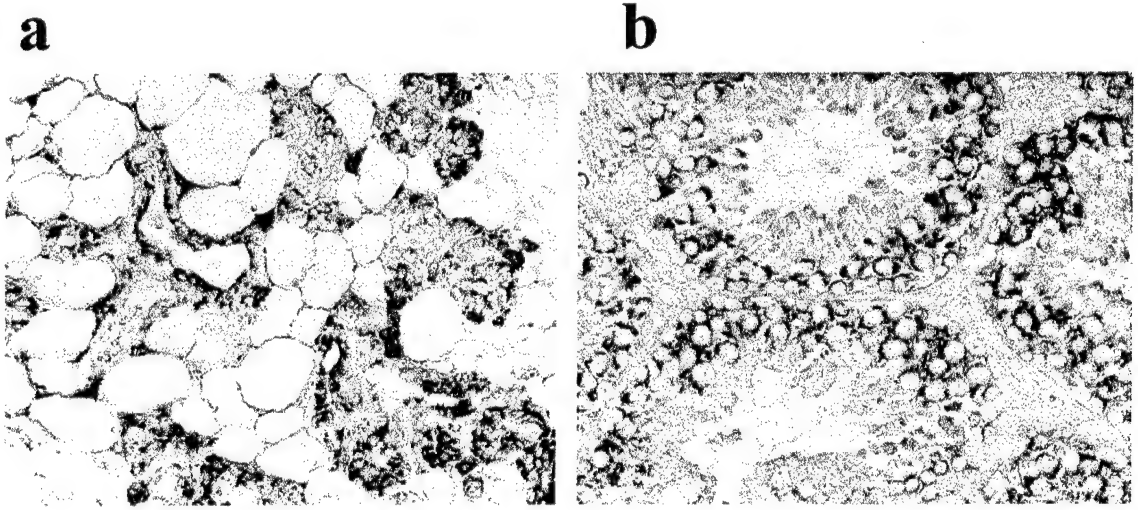
Electromicrographs of mammary tissue of transgenic mice expressing UOX under the control of MMTV promoter, showing the presence of UOX in the form of crystalloid structures.

Fig.9



Immunohistochemical analysis: Mammary and testicular tissue from transgenic mice expressing rUOX as confirmed by northern blot analysis, were fixed and processed for immunohistochemical analysis. Frozen sections on slide, were treated for antigenic exposure and then probed with anti UOX antibody after blocking with goat serum. Secondary antibody coupled to rhodamine was used to detect the expression of UOX in (a) mammary tissue and (b) testicular tissue. The expression of rUOX can be seen by the red color

Fig. 10



In situ hybridization: Tissues of mammary and testicular region were fixed and processed for in situ hybridization. Sense and anti sense probes derived from the UOX cDNA were hybridized to the RNA present in the tissues. The expression of rUOX can be seen in blue color in (a) mammary and (b) testis.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of plasmids for in vitro expression
- Construction of MMTV-UOX transgene for the generation of transgenic mice
- Microinjection of transgene into fertilized ova and generation of 14 founder mice.
- Founders were bred with wildtype and germ-line transgenics were established.
- Heterozygous mice containing rUOX were identified by Southern analyses
- Southern positives were screened for RNA expression by Northern analyses
- 7 heterozygous mice from 3 founders were found to be RNA positive
- RNA positive mice were also screened for protein expression and identified by Immunoblot analyses.
- Transgenic mice were screened for the expression of rUOX using immuno histochemical analysis and the protein expression was found to be uniformly localized in the cytoplasm of epithelial cells of the mammary tissue and the germinal cells of the testicular cells.
- Transgenic mice were also screened for rUOX using in situ hybridization, to accurately establish the presence of rUOX mRNA. Sense and antisense probes of rUOX cross reacted and localized the expression of rUOX in the epithelial cells of mammary tissue and the germinal cells of testicular tissue.
- Stable cell lines expressing rUOX under the control of MMTV promoter and CMV promoter are developed to increase the expression of rUOX and to inject them to nude mice.

REPORTABLE OUTCOMES

- Review entitled "Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis" by Yeldandi, A.V., Rao, M.S., and Reddy, J.K. *Mutation Research* 448:159-177, 2000

CONCLUSIONS

We have successfully generated heterozygous mice and confirmed that they have a functional transgene (UOX), which was confirmed by Southern blot, Northern blot and Immunoblot analyses. Expression and localization of rUOX was established by immuno histochemical analysis as well as by in situ hybridization. Stable cell lines expressing rUOX under the control of MMTV and CMV promoter are also developed. We will initiate studies to examine the role of reactive oxygen species in cell death, cell proliferation and neo plastic transformation in mammary cells expressing urate oxidase

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